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**This is a pre print version of the following article:**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1724381> since 2021-01-31T11:36:23Z

*Published version:*

DOI:10.1094/PDIS-01-19-0035-RE

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(Article begins on next page)

1    **Rapid Detection of *Monilinia Fructicola* And *Monilinia Laxa* on Peaches and Nectarines Using**  
2    **Loop-Mediated Isothermal Amplification**

3

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## 26    **Abstract**

27    *Monilinia laxa* and *Monilinia fructicola* are two causal agents of brown rot, one of the most important  
28    diseases in stone fruit. Two species cause blight on blossoms and twigs, and brown rot on fruits in  
29    pre- and postharvest. Both species are worldwide distributed in North and South America, Australia  
30    and Japan. In Europe, *M. laxa* is endemic, while *M. fructicola* was introduced in 2001 and it is now  
31    widespread in several countries. Currently, both species are coexisting in European stone fruits  
32    orchards. *Monilinia* spp. overwinter in cankers and mummified fruits. Mummy monitoring during  
33    winter permits to understand which species of *Monilinia* will be prevalent in orchard during the  
34    following season, permitting to plan an appropriate crop protection. Traditionally, the identification  
35    has been carried out using morphological features and even with PCR-based assays that requires time  
36    and well-equipped laboratories. In this study, two isothermal-based methods were designed to  
37    identify these pathogens in a faster way than using traditional methods. The Loop-mediated  
38    AMPlification (LAMP) assays were validated on some isolates of *Monilinia* spp. coming from the  
39    mummy monitoring according to the international EPPO standard (PM7/98) taking into account the  
40    specificity, sensitivity, repeatability and reproducibility. The sensitivity of both assays was checked  
41    by monitoring at different time points two nectarines varieties artificially inoculated and stored at two  
42    different temperatures. The reliability of both LAMP assays against the quantification of the inoculum  
43    was compared with previously published qPCR assays. Both LAMP methods were able to detect low  
44    number of cells. These LAMP methods could be a useful tool for the monitoring brown rot causal  
45    agents in the field and during postharvest.

46  
47    **Keywords:** LAMP, brown rot, *Prunus persica*, nectarine, peach, field, molecular diagnostics

*Monilinia laxa* (Aderhold and Ruhland), *Monilinia fructicola* (Winter) Honey, *Monilinia fructigena* (Adehold and Ruhland) and *Monilia polystroma* Leeuwen are the main agents of brown rot, one of the most important diseases in *Prunus*, *Malus* and *Pyrus* species (Batra 1991).

Brown rot is particularly serious in peach and nectarine production by causing blossom and twig blights and brown rot on the fruits at preharvest, harvest and postharvest (Hong et al. 1997). Brown rot losses can reach 90%, by considering the harvest and postharvest stages (Hong et al. 1997; Hong et al. 1998). However, the postharvest stage is the most critical one, due to the optimal environment for *Monilinia* growth (Harvey 1978; Eckert and Ogawa 1988). The most important and widespread species on peaches and nectarines are *M. laxa* and *M. fructicola*. *M. fructicola* was originally identified in North and South America, Australia and Japan (EPPO/CABI 1997) and was introduced in Europe (France) on peaches in 2001 (Lichou et al. 2002). This pathogen is now spread throughout Europe, with reports in Austria (OEPP/EPPO 2002), Hungary and Spain on peaches (De Cal et al. 2009), Italy on nectarines (Pellegrino et al. 2009), Germany on blackberries and plums (Hinrichs-Berger and Müller 2010), Slovenia on peaches (Munda and Viršček Marn 2010), and Poland on apples, pears and plums (Poniatowska et al. 2013). In 2005, *M. fructicola* was included in the A2 EPPO List of quarantine organisms due to the high yield losses reported on peaches, apricots and nectarines in Europe (EFSA 2011). On the other hand, *M. laxa* is a quarantine pathogen in China and in some parts of North America (Martini and Mari 2014) and was the most prevalent species in Europe until the introduction of *M. fructicola*. Currently, both species are coexisting in European stone fruits orchards (Villarino et al. 2013).

*M. fructigena*, instead, is more prevalent in pome fruits and it has a low incidence in stone fruits (Martini and Mari 2014). *M. polystroma*, morphologically similar to *M. fructigena*, was initially identified in Japan (Côté et al. 2004) and has been introduced in Europe with reports in apples in Hungary (Petróczy and Palkovics 2009) and Croatia (Di Francesco et al. 2015), in apricots in Switzerland (Hilber-Bodmer et al. 2012), and in peaches in Italy (Martini et al. 2014). However, this

73 species is less aggressive and less prevalent, similarly to other minor species, such as *M. numecola*  
 74 and *M. tunnanensis* (Cox et al. 2018).

75 In the United States, the stone fruit market has an annual value of approximately 4.4 billion  
 76 \$ (Cox et al. 2018). Brown rot is mainly caused by *M. fructicola*, the most predominant in stone  
 77 fruits, while *M. laxa* is present in some areas of North America with lower incidence (Cox et al.  
 78 2018). However, *M. laxa* causes important losses due to development of blossom and shoot blights  
 79 on almonds, apricots, nectarines and cherries (Cox et al. 2011).

80 *Monilinia* spp. overwinter in cankers on branches and on the mummified fruits remaining  
 81 from one year to the next one. Mummy monitoring during winter permits to understand which species  
 82 of *Monilinia* will be prevalent in orchard during the following season, permitting to plan an  
 83 appropriate crop protection. Ascospores produced on the apothecia of infected mummified fruits are  
 84 an important source of inoculum that causes primary infections or blossom blights in the early spring  
 85 (Tate and Wood 2000).

86 Due to the importance of brown rot caused by *Monilinia* spp., intensive disease management  
 87 is key to reduce blossom blight during spring and to reduce the risk of fruit infection. Different  
 88 sensitivity to fungicides, such as benzimidazoles and dicarboximides (Chen et al. 2013; Egüen et al.  
 89 2016), has been reported for different species of *Monilinia*. The use of fungicides during preharvest  
 90 is the most common measure used to control the incidence of pre and postharvest brown rot (Eckert  
 91 and Ogawa 1988, Thomidis et al. 2009), however the decision about the optimal moment for chemical  
 92 control is based on forecasting models. The request of reducing the use of fungicides is boosting the  
 93 development of alternative crop protection strategies (Malavolta et al. 2003), including biocontrol  
 94 agents (Larena et al. 2005; Zhang et al. 2010; Banani et al. 2015) or natural substances (Mari et al.  
 95 2008; Lopez-Reyes et al. 2013; Santoro et al. 2018).

96 A correct identification of the species of *Monilinia* may improve crop protection. Fungal  
 97 isolation and micromorphological analysis are necessary to identify the species of *Monilinia*, but  
 98 morphological features may vary depending on the incubation media and conditions making difficult

99 an accurate species-specific identification (Byrde 1977, Lane 2002). In addition, micromorphological  
 100 identification requires a long incubation period that is critical for decision making (OEPP/ EPPO  
 101 2009). Different assays based on PCR amplification of rDNA internal transcribed spacer region (Ioos  
 102 and Frey 2000), RAPD (Boehm et al. 2001; Côté et al. 2004), PCR with SCAR primers (Hughes et  
 103 al. 2000), microsatellites using a nested-PCR (Ma et al. 2003; Boehm et al. 2001) or qPCR (Van  
 104 Brouwershaven et al. 2009; Guinet et al. 2016, Wang et al. 2018) have been developed to differentiate  
 105 the species of *Monilinia*. However, these methods are time-consuming and require transport to a  
 106 diagnostics laboratory, which delays the decision making process.

107 Loop-mediated isothermal AMPlification (LAMP) assay demonstrated to be a specific,  
 108 sensitive and reliable tool for fungal identification in routine diagnostics (Franco Ortega et al. 2018a).  
 109 The LAMP reaction is an isothermal DNA amplification method with an enzyme copying the target  
 110 region faster than other PCR based methods, whilst avoiding the use of thermal cycling (Notomi et  
 111 al. 2000; Nagamine et al. 2002). The enzyme is also robust, thus complex DNA extraction is not  
 112 required to perform the LAMP assay, which is not affected by inhibitors. The greatest advantage of  
 113 the LAMP assay is the possibility of using battery-powered platforms, such as Genie II or Genie III  
 114 (Optigene, Horsham, UK), which make the LAMP a suitable diagnostic tool for direct detection in  
 115 field, packinghouse or during transport and storage stages.

116 The aim of the present study was to monitor the occurrence of the emerging pathogen *M.*  
 117 *fructicola* and other species of *Monilinia* spp. in peach and nectarine orchards during the first four  
 118 consecutive years of occurrence in Italy. Mummy monitoring during winter time permits to plan an  
 119 appropriate crop protection strategy against brown rot in the following growing season. A collection  
 120 of isolates of *Monilinia* spp. was created, by isolating from peach and nectarine mummies harvested  
 121 at the end of the winter season. The strains were identified morphologically and with molecular tools.  
 122 The collection constituted the basis for developing two LAMP assays, one for *M. fructicola* and the  
 123 other one for *M. laxa*, the two main species of *Monilinia*. The molecular assays were validated on a  
 124 selection of isolates from the mummy collection and on inoculated nectarines.

125

126 **Materials and Methods**

127 **Pathogen isolation.** Forty-six peach and nectarine fields listed in Table S1 were monitored  
 128 during the winter seasons from 2008-2009 to 2011-2012 to verify the occurrence of *Monilinia*  
 129 *fructicola* and of other species of *Monilinia*. The monitoring was carried out on twenty cultivars of  
 130 peach and nectarine: ‘Alitop’, ‘Amiga’, ‘Big Top’, ‘Caldesi’, ‘Diamond Ray’, ‘Elegant Lady’,  
 131 ‘Firebrite’, ‘Fire Top’, ‘Fire Sweet Red’, ‘Maeba Top’, ‘Nectaross’, ‘Orion’, ‘Red Valley’, ‘Rome  
 132 Star’, ‘Royal Glory’, ‘Sweet Lady’, ‘Stark Red Gold’, ‘Vista Rich’, ‘Venus’, and ‘V3’ (Table S1).  
 133 Twenty-five mummified fruits, when present, were collected from each orchard, disinfected in a  
 134 sodium hypochlorite (1%) solution, rinsed under tap water and dried. Small pieces of infected  
 135 material were placed on Potato Dextrose Agar (PDA; Sigma-Aldrich, Saint Louis, MO, USA) to  
 136 isolate the pathogen. The samples were grown at 23-25°C for four days. Single spore cultures of each  
 137 *Monilinia* isolates were obtained and stored in 60% glycerol at -80°C. The isolates were stored at  
 138 Agroinnova microorganism collection (University of Turin, Italy). For the development of the LAMP  
 139 assays, besides the isolates of *Monilinia* spp. coming from the field monitoring, some *Monilinia* spp.  
 140 from the FERA collection (York, UK) and two isolates of *M. polystroma* provided by the University  
 141 of Bologna were used.

142 **Fungal isolates and DNA extraction.** Single spore isolates listed in Table 1 were grown on  
 143 PDA for 10 days at room temperature. Mycelium was collected and the total genomic DNA was  
 144 extracted with E.Z.N.A. Fungal DNA mini kit (OMEGA Bio-Tek, Norcross, GA, USA), according  
 145 to the manufacturer’s instructions. The DNA concentration of each isolate was measured using a  
 146 Nanodrop 1000 (ThermoFisher, Delaware, USA) and the concentration of DNA was adjusted at 1 to  
 147 50 ng/μl. A multiplex PCR developed by Côté et al. (2004) with the primers listed in Table S2 was  
 148 used to identify all the samples obtained during the monitoring. Fungal species identification was  
 149 confirmed with the primers designed by Hughes et al. (2000), including the primer ITS1 for *M.*

150 *fructigena*, and the primers described by Gell et al. (2007). Amplified products were checked on 1.5%  
 151 agarose gel stained with ethidium bromide.

152 **Crude extraction method from peaches.** DNA was extracted from artificially inoculated  
 153 peaches using a crude extraction method to avoid the use of long and complex procedures. The crude  
 154 extraction was based on the procedure described by Chomczynski and Rymaszewski (2006) and  
 155 Tomlinson et al. (2010a). Alkaline PEG buffer (1 ml) composed of 20 mM KOH at pH 13.5 with 50  
 156 g L<sup>-1</sup> of PEG average of Mm: 4,600 a with 1 ball bearing (7/16" stainless steel 316 GD Spheric  
 157 Trafalgar Ltd) in a 5 ml tube was used for the crude extraction by adding 1-2 g of the inoculated peach  
 158 material (adding the skin and flesh of the inoculated point, cutting by a sterile scalpel). Samples were  
 159 manually shaken for one minute. The solution was ten-fold diluted to use in the LAMP reaction. The  
 160 peach genomic DNA was also obtained in parallel with E.Z.N.A. Plant DNA kit (OMEGA Bio-Tek)  
 161 to compare the results from both DNA extraction methods.

162 **LAMP primer design.** Six LAMP primers including two external primers, F3 and B3; two  
 163 internal primers, FIP (F1c+ F2) and BIP (B1c+B2); and two loop primers, Floop and Bloop, were  
 164 designed from *M. fructicola* and *M. laxa* sequences according to the method described by Notomi et  
 165 al. (2000). The LAMP primers for *M. fructicola* were designed on an intron in the cytochrome b,  
 166 associated with the Qol fungicides resistance, present in *M. fructicola* (GenBank accession number  
 167 GQ304941.1), but absent in other *Monilinia* species (Luo et al. 2010). The primers for *M. laxa* were  
 168 designed on a genomic sequence identified as a SCAR marker by Gell et al. (2007) (GenBank  
 169 accession number: EF207417.1). All the primers were manually designed taking into account the  
 170 annealing temperature. Possible hairpin and secondary structures were checked using the OligoCalc  
 171 program (<http://biotools.nubic.northwestern.edu/OligoCalc.html>), while possible interactions  
 172 between primers was controlled using the Multiple Primer Analyzer (Thermo Scientific)  
 173 ([https://www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-](https://www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html)  
 174 [primer-analyzer.html](https://www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html)). HPLC-purified primers were synthesized by Eurofins (UK) and both LAMP  
 175 Davide Spadaro  
 Plant Disease



assays are available in kit format from OptiGene Ltd (Horsham, UK: <http://www.optigene.co.uk>). BLASTn analysis of the F1c and B1c of both primers was performed to check possible cross-reactivity *in silico*.

**LAMP assay.** The LAMP reaction (25 µl) contained 200 nmol/l of each external primer (F3 and B3), 2 µmol/l of each internal primers (FIP and BIP), 1 µmol/l of each loop primer, 1x Isothermal Mastermix ISO-004 (OptiGene Ltd) with 1 µl of the pure DNA or the crude extractions. A Genie II ® instrument (OptiGene Ltd) and a StepOne (Applied Biosystem, California, USA) was used to carry out the LAMP tests. The program conditions were 45 min at 65°C and a measure of the annealing temperature from 95°C to 70°C with a reduction of 0.05°C/s. The StepOne was programmed as described previously by Franco Ortega et al. (2018b). A negative control with water and a positive control with the pure DNA of the target were included in each assay. The COX assay described by Tomlinson et al. (2010b) was applied as internal control to verify if the plant DNA from the samples resulted negative with the LAMP assays for *M. fructicola* and *M. laxa* could be amplified.

**Validation of the LAMP assays.** The LAMP assays were validated according to the international standard EPPO PM 7/98 taking into consideration specificity, sensitivity, repeatability and reproducibility. The specificity of the assays were checked in triplicate using an inclusivity-exclusivity panel of the target and non-target *Monilinia* spp., as well as other common pathogens of stone fruits. The sensitivity was tested on 10-fold serial dilutions of the DNA from one isolate of *M. fructicola* (isolate MSR38) and one isolate of *M. laxa* (isolate 1406) with four replicates of each dilution. The extraction and the LAMP assays were performed on different days with different machines (Genie II and StepOne) by three members of the lab staff to evaluate the reproducibility. The reliability of both LAMP assays was confirmed in parallel with a qPCR assay on two cultivars of nectarines, using three biological replicates and three technical replicates for each assay.

**Artificial inoculation and LAMP assay on fruit.** Nectarines ‘Amiga’ and ‘Fire Top’ were inoculated with *M. fructicola* MSR2 and *M. laxa* 1506. Healthy nectarines were disinfected by submerging in 2% hypochlorite (vol: vol) solutions before being wiped with distilled water and air-

202 dried. The fruit inoculation was performed in artificial wounds (2 mm in diameter and 4 mm deep)  
 203 using 10 µl of the spore suspension ( $10^5$  conidia/ml) prepared from 10-day old *Monilinia* cultures  
 204 grown on PDA. The fruits were stored at 18°C or at 4°C. The DNA from the inoculation region of  
 205 the fruit was used in the crude extraction method and the E.Z.N.A. Plant DNA kit. The quantity of  
 206 the fruit tissue of each nectarine used in each extraction was measured to calculate the number of  
 207 cells present in the sample. The LAMP assay was carried out with the crude extraction method at 2  
 208 days post inoculation (dpi), 4/5 dpi, 7 dpi, 9 dpi, 11/12 dpi for the nectarines stored at 18°C and at 2  
 209 dpi, 4/5 dpi, 7 dpi, 9 dpi, 11/12 dpi, 14 dpi and 16 dpi for the nectarines stored at 4°C. Sampling times  
 210 were 4 and 11 dpi for 'Amiga' and 5 and 12 dpi for 'Fire Top'. The fruit symptoms were also scored  
 211 using the following disease index: 0: no evident symptoms; 1: brown rot lower than 1 mm diameter;  
 212 2: brown rot of 1-3 mm diameter; 3: brown rot of 1-2 cm diameter; 4: at least half of the fruit surface  
 213 presented brown rot; 5: mummified fruit. The disease severity was calculated using the average of  
 214 three replicates (the same 3 nectarines selected randomly from the inoculated batch and posteriorly  
 215 used for both DNA extractions). Samples prior to inoculation were used in both LAMP and qPCR  
 216 analysis as negative controls.

217 **Real-Time PCR.** The primers designed by Hughes et al. (2000), Mcf-F1 and Mfc-R1 specific  
 218 for *M. fructicola* and MI-Mfg-F2 and MI-Mfc-R1 specific for *M. laxa*, were used to quantify the  
 219 number of cells present in the samples. The 25 µl reactions were carried out using 1x Power SYBR  
 220 Green PCR Master Mix (Applied Biosystems), 120 nM of each primer and 1 µl of the peach genomic  
 221 DNA extracted using the commercial kit. The amplification was carried out using the following  
 222 protocol: 95°C for 10 min, 40 cycles of 15 s at 95°C, 1 min at 60°C, and 45 s at 72°C in a OneStep  
 223 Plus Real-Time PCR system (Applied Biosystems). A standard curve was performed using *M.*  
 224 *fructicola* MSR2 and *M. laxa* 1506 ranging from 9.4 ng/µl to 9.4 fg/µl. A positive control with DNA  
 225 extracted from cultured pathogen and a negative control of water were included in each run. The  
 226 quantity of DNA present in the sample was calculated according to the standard curve measures,  
 227 while the number of cells was estimated using the *Monilinia fructicola* genome weight (0.000048 ng;

228 <https://www.ncbi.nlm.nih.gov/Traces/wgs/?val=NGKE01#contigs>) using the formula: number of  
 229 cells / $\mu\text{l}$  = DNA quantity/0.000048 (Amaral Carneiro et al. 2017). The amount of fruit used (ranging  
 230 from 1 g to 2 g) and the elution volume of the extracted DNA were used to calculate the total number  
 231 of cells.

232 **Analytical specificity and sensitivity assays.** The inoculated nectarines (24 samples) and  
 233 eight negative samples (four for either cultivar) were used to calculate some analytical parameters for  
 234 both LAMP assays, which were compared with the SYBR Green qPCR of Hughes et al. (2000).  
 235 Diagnostic sensitivity (DSe) or true-positive rate and analytical specificity (DSp) or true-negative  
 236 rate, were calculated using the formula as follows:  $Dse = \frac{\sum TP}{(\sum TP + FN)}$ ;  $Dpe = \frac{\sum TN}{(\sum TN + FP)}$ ;  
 237 where TP (True Positive) is the number of positive samples in the experiment, TN (True Negative) is  
 238 the number of real negative samples, FN (False Negatives) are the negative samples using the LAMP  
 239 assay but infected with *Monilinia* spp., and FP (False Positives) are the positive results using LAMP  
 240 assay and naturally free from pathogens (Altman and Bland, 1994). The likelihood positive ratio  
 241 (LR+) and the likelihood negative ratio (LR-) were calculated according to the ratio between DSe and  
 242 the FP-rate and the FP-rate and Dse, respectively, using the calculator tool Diagnostic Test Calculator  
 243 (<http://araw.mede.uic.edu/cgi-bin/testcalc.pl>)

244

## 245 Results

246 **Field monitoring.** During the monitoring, 1889 mummified fruits were harvested (281 in  
 247 2008/09, 480 in 2009/2010, 542 in 2010/2011, and 586 in 2011/2012). The number of isolates of the  
 248 different species of *Monilinia* were recorded to get the incidence in the orchards: 200 isolates from  
 249 the mummies of 2008/09, 417 from 2009/10, 396 from 2010/11, and 470 from 2011/12. This  
 250 monitoring permitted to identify the evolution over time of the species of *Monilinia* spp. from the  
 251 mummies, able to cause brown rot in peaches and nectarines.

252 An increase in the occurrence of *M. fructicola* was shown during the years, from a minimum of 6.2%  
 253 during the 2008-2009 winter season to a maximum of 49.6% in the 2011-2012. On the other hand,  
 254 the incidence of *M. laxa* decreased from 91.8% in 2008-2009 to 46.6% in 2011-2012. In the 2011-  
 255 2012 winter season, the incidence of *M. fructicola* overtook the incidence of *M. laxa*. (Figure 1). The  
 256 incidence of *M. fructigena* was constantly low and did not vary significantly during the study period:  
 257 from 2.0% (2008-09), to 2.7% (2009-10), 2.1% (2010-11) and 3.8% (2011-12).

258 *M. fructicola* was more abundant in peaches than in nectarines during the winter season 2011-2012  
 259 (Figure 2). Among the cultivars analyzed during the last winter season, ‘Fire Top’ (90%), ‘Fire Sweet  
 260 Red’ (90%), ‘Rome Star’ (76.1%), ‘Diamond Ray’ (69.1%), ‘Alitop’ (63.35%), and ‘Nectaross’  
 261 (61.1%) showed the highest occurrence of *M. fructicola*, while the varieties with the highest incidence  
 262 of *M. laxa* were ‘Stark Red’ (100%), ‘Caldesi’ (69.2%), ‘Royal Glory’ (66.7%) and ‘Big Top’  
 263 (56.9%). The highest incidence of *M. fructigena* was found in the variety ‘Caldesi’ (30%), while in  
 264 the other varieties the incidence was just 7%.

265 Variation in the *Monilinia* species present was found in different areas. All samples from three  
 266 locations (Dronero, Manta and Piasco; Piedmont, northern Italy) were identified as *M. laxa*, while *M.*  
 267 *laxa* was not isolated from the samples from Castellar and Scarnafigi. The other areas showed  
 268 different percentages of *M. laxa* and *M. fructicola*. *M. fructigena* was present in less than 8% of the  
 269 samples tested (Figure S1).

270 A collection of isolates of *Monilinia* spp. was created, useful for the development of a new diagnostic  
 271 method to distinguish the predominant species of *Monilinia*. A significant number of isolates from  
 272 the monitoring were used in the specificity test of both LAMP assays.

273 **Design and validation of the LAMP assay using DNA from pure culture.** The primers for  
 274 the identification of *M. fructicola* and *M. laxa* were designed on the cytochrome b sequence and on  
 275 the SCAR marker region, identified by Gell et al. (2007), respectively. All primers were checked for  
 276 lack of secondary structure, self-annealing or hairpin.

BLASTn analysis of the F1c and B1c (primer FIP and BIP) for the detection of *M. fructicola* showed 100% sequence identity with *M. fructicola* (GenBank accession number KM610206.1), while the F1c and B1c regions of the FIP and BIP primers for the detection *M. laxa* had 100% identity to the sequence of this species (accession number EF207417.1).

Initially, identification of the *Monilinia* spp. was carried out using the primers designed by Hughes et al. (2000) and by Gell et al. (2007). Both PCR analyses confirmed the results of the LAMP assays. The isolates of *Monilinia* spp. from the monitoring, as well as other isolates, were used to validate the specificity of the LAMP assays, which is reported in Table 1. The LAMP primers amplified only the targets (*M. fructicola* and *M. laxa*, respectively), while untargeted amplifications were not obtained. The time to positive (Tp) for the *M. fructicola* LAMP assays ranged from 7 min 35 s to 25 min, with an annealing temperature ranging from 84.06°C to 85.54°C. For the *M. laxa* LAMP assay the Tp ranged from 7 min 11 s to 24 min 26 s, with an annealing temperature ranging from 81.52°C to 85°C.

The sensitivity testing demonstrated that the *M. fructicola* LAMP assay was able to detect 100-999 fg of DNA, while the *M. laxa* LAMP assay had a lower detection limit of 10-99 fg of DNA (Table 2). The assays consistently showed positive results in the three replicates tested, even by changing the machine, the operator, or the day of the test.

***In vivo* testing.** Both LAMP assays were tested on two varieties of nectarines. Brown rot symptoms were visible earlier on ‘Amiga’ than on ‘Fire top’ nectarines at both temperatures. ‘Fire top’ nectarines inoculated with *M. fructicola* and stored at 18°C started to exhibit concentric lesions from the inoculation area with evident symptoms of brown rot at 7 dpi (Table 3). Instead, ‘Amiga’ nectarines stored at 18°C exhibited symptoms at 4 dpi (Table 4). Nectarines inoculated with *M. fructicola* and stored at 4°C showed the first symptoms of brown rot at 14 dpi on ‘Fire Top and at 7 dpi on ‘Amiga’. Brown rot symptoms were visible earlier by inoculating *M. laxa* than *M. fructicola* on both varieties.

302 The LAMP assay for *M. fructicola* was able to detect the presence of the pathogen in both nectarine  
303 varieties before the appearance of brown rot symptoms (Table 3 and 4). The Tp ranged from 4 min  
304 33 s to 15 min from the DNA of nectarines stored at 18°C, and ranged from 5 min 52 s to 36 min,  
305 with the majority of the positive results between 9-13 min, from the DNA of nectarines stored at 4°C  
306 (Tables 3 and 4). The annealing temperature ranged from 84.35 to 85.18°C (Tables 3 and 4).

307 The *M. laxa* LAMP assay gave positive results prior to symptom development in both nectarine  
308 varieties and at both storage temperatures (Tables 5 and 6). Anyway, there were differences in the  
309 LAMP assay for *M. laxa* between the varieties: ‘Fire Top’ nectarines were not positive until 7 dpi at  
310 both storage temperatures (Table 5), while in ‘Amiga’ nectarines the pathogen was detected at 2 dpi  
311 (Table 6). Using the DNA from *M. laxa*-inoculated ‘Amiga’ nectarines stored at 18°C, the LAMP  
312 assay produced a Tp ranging from 4 min 2 s to 24 min 15 s (Table 6). The Tp of the nectarines stored  
313 at 4°C ranged from 4 min 2 s to 28 min 12 s. The annealing temperature ranged from 83.15 to 83.55°C  
314 when all samples were compared (Tables 5 and 6).

315 **Comparison between qPCR and LAMP assay.** The number of cells present during the  
316 experiments were quantified using SYBR Green and the combination of primers obtained by Hughes  
317 et al. (2000) (Tables from 3 to 6). In order to check the sensitivity of the LAMP assays, the number  
318 of cells of three nectarines were compared with an average of the disease index of the same nectarines  
319 to obtain a real comparison between molecular data and symptoms. The number of cells increased  
320 over time reaching a maximum of 20,000,000 cells for the variety ‘Amiga’ stored at 18°C. The  
321 number of cells of the nectarines increased day after day, similarly with the disease index. This trend  
322 was observed in both varieties (‘Amiga’ nectarines inoculated with *M. fructicola* and *M. laxa* stored  
323 at 18°C and 4°C respectively, as well as ‘Fire Top’ inoculated with *M. laxa* and stored at 18°C),  
324 however the LAMP assay gave reliable detection.

325 Different results were obtained with the qPCR and the LAMP assays. In the case of *M.*  
326 *fructicola*-inoculated nectarines, both techniques showed positive results for both nectarine varieties  
327 at both storage temperatures, with only one exception in which the qPCR was not able to detect any

cells ('Fire Top' nectarines at 2 dpi). However, as observed in the Table 3 and 4, there were significant differences between the number of positives obtained with each technique, being the qPCR more repeatable.

On the other hand, there were bigger differences between the qPCR and the LAMP assays to detect *M. laxa*. In the case of 'Fire Top' nectarines, the nectarines stored at 18°C at 2 dpi and the nectarines stored at 4°C at 2 and 4 dpi, were only detected with the qPCR and not with the LAMP (Table 5). However, 'Amiga' nectarines stored at both temperatures were detected with the LAMP at all time points, but the qPCR produced negative results in two cases: 18°C stored nectarines at 2 dpi and 4°C stored nectarines after 9 dpi (Table 6). The LR+ and LR- of both assays were calculated to check the effectiveness of the LAMP assay in comparison with the qPCR with SYBR Green. In the case of the LAMP assay for *M. fructicola*, the value of Dsp and Dse were 1.0 (100%), while the Dsp and Dse values of the specific primers were 0.88 (88%) and 0.96 (96%) respectively. The *M. laxa* LAMP assay showed a DSp value of 0.83 (83%) and a DSe value of 0.67 (67%) compared with the 0.88 (0.88%) and 0.73 (73%) of the DSp and Dse values of the specific primers.

Taking into consideration the likelihood ratios, the LR+ and LR- of *M. fructicola* LAMP assay were infinite, while the LR- of the *M. laxa* LAMP assay was 0.17 and the LR+ was infinite. The same values calculated for the qPCR primers with SYBR Green for *M. fructicola* and *M. laxa* showed in both cases infinite LR+ and LR- of 0.04 and 0.12, respectively.

## Discussion

The occurrence of *Monilinia* spp. was monitored during four winter seasons in Italian peach and nectarine orchards for a better understanding of the establishment of the introduced species *M. fructicola* and of the evolution of the species of *Monilinia* spp.. The results of the mummy monitoring over 4 years helped to understand the capacity of coexistence of *M. laxa* and *M. fructicola* in the same orchards. Since the first report of *M. fructicola* in Europe, the pathogen, listed as an EPPO A2

353 pathogen due to the damage caused (OEPP/EPPO 2005), has spread to different countries. Despite  
 354 the importance of stone fruit production in Italy, an extensive monitoring activity has not not  
 355 performed by other authors since the first occurrence (Pellegrino et al., 2009). The geographical  
 356 region where *M. fructicola* was first identified in Italy, was the focus of this study. *M. laxa* and *M.*  
 357 *fructicola* could produce fruit losses up to 90% (Larena et al. 2005; Hong et al. 1997), under favorable  
 358 weather conditions. The losses caused by *M. fructigena* are by far lower, with a relatively low  
 359 incidence in Italy. In this study, the incidence of *M. fructicola* increased year after year, overtaking  
 360 the incidence of *M. laxa* in the 2011-12 survey. This suggested a higher fitness of *M. fructicola* and  
 361 the higher adaptability to the weather conditions of northern Italy.

362 The estimation of the mummy inoculum and the optimal environmental conditions for the  
 363 disease development should be considered to understand the seasonal variation of the disease and to  
 364 ensure the timely implementation of a management strategy against brown rot (Luo et al. 2001a; Luo  
 365 and Michailides 2001a). The monitoring of mummies during winter permits to plan an appropriate  
 366 crop protection strategy, which depends on the species of *Monilinia* present in the orchard. An  
 367 Integrated Pest Management (IPM) approach is suggested by EFSA (2011) in order to effectively  
 368 control brown rot and blossom blight, focusing on reducing the inoculum of *Monilinia* in the field  
 369 and therefore the risk of infection (Luo and Michailides 2001b). The importance of the mummies as  
 370 an important inoculum source was confirmed during the survey. Therefore, removal and destruction  
 371 of the fruit mummies is a key aspect of the sanitation process, especially given the diffusion speed of  
 372 brown rot in orchards.

373 During postharvest, the incidence of brown rot is associated with the incidence of *Monilinia*  
 374 spp. in orchard. Any delay on the application of control measures for *Monilinia* spp. can cause losses  
 375 of over 50% particularly during postharvest, but yield losses can be reduced if correct control  
 376 measures are timely applied (Margosan et al. 1997). However, some of the measures developed to  
 377 reduce brown rot have been demonstrated to be species-dependent (Mari et al. 2012; Lopez-Reyes et  
 378 al. 2013), making necessary a precise identification of the causal agent of brown rot. Until now, no



LAMP tests have been developed to directly check the presence of *Monilinia* on fruit, therefore the methods described in this study could help growers to control the presence of brown rot in peaches, by supporting the choice of the correct control measures before harvesting and during storage. One of the biggest drawbacks of the PCR-based methods is the inhibition of the reaction by some components of plant tissue which results in false negative results (Wilson 1997), therefore most PCR-based methods involve long and complex DNA extraction methods. As an alternative, the LAMP assays for the detection of *M. fructicola* and *M. laxa* were validated using a crude extraction method, which is simpler, and showed to be reliable and effective in the detection of both pathogen species. The validation of the assays was carried out according to the international standard published by EPPO (PM7/98). A significant number of isolates from the monitoring were used to validate the specificity of both LAMP assays. To verify the parameters of both LAMP assays *in vivo*, we focused on fully ripe nectarines, which were stored at two temperatures reflecting the storage and shelf life conditions of stone fruit.

The LAMP assay for *M. fructicola* was less sensitive than the LAMP assay for *M. laxa*. Notwithstanding, on nectarines inoculated with different amounts of *M. laxa*, the pathogen detection was possible even without visible symptoms. The LAMP results were confirmed using qPCR. The lowest number of cells in all the ‘Fire Top’ nectarines inoculated with *M. fructicola* was 259.7 cells (no symptoms visible) giving two positive replicates out of nine with the qPCR and six out of nine with the LAMP assay, which verified the reliability of the assay. In a similar situation, *M. laxa* (73.5 cells) produced less positive results with the LAMP assay (1/9 compared to 2/9 with the qPCR). In contrast, ‘Amiga’ nectarines were less susceptible to *M. fructicola* and *M. laxa* with less cells quantified and lower symptoms in all the experiments. The use of the LAMP assay on the ‘Amiga’ variety confirmed the reliability and effectiveness of the LAMP assays on the detection of few cells (22.8 cells and even 1.3 cells) independently from the nectarine variety, suggesting that the assay is potentially a powerful tool for pre-symptomatic detection.

404 According to the natural disease development, a consistent and constant increase in the  
 405 number of pathogen cells was expected over time, however, in some cases, the pattern of the disease  
 406 did not correspond with the number of cells shown. In particular, when the whole fruit showed brown  
 407 rot or the nectarines were mummified, the number of cells were not the highest ones. This could be  
 408 explained with the high amount of fungal DNA present in the sample, especially in the mummified  
 409 fruits, which could inhibit the qPCR. However, the LAMP assay reliably detected the pathogen even  
 410 in these cases.

411 On the other hand, the LAMP assays were able to detect the presence of the pathogen even  
 412 without visible symptoms, when the qPCR did not produce positive results. In summary, both LAMP  
 413 assays were validated and could be reliably used for the detection of *M. fructicola* and *M. laxa*.  
 414 Despite the use of a crude extraction method instead of a commercial and complex DNA extraction  
 415 used for the qPCR assay, the results of the LAMP assay were consistent, and no inhibition or loss of  
 416 sensitivity were reported taking into consideration the likelihood ratios which were similar to those  
 417 of the qPCR for both pathogens. The monitoring performed during four winter seasons suggested a  
 418 higher adaptability of *M. fructicola*, compared to *M. laxa*, in the Italian orchards. The LAMP assays  
 419 allow a faster identification in orchard of *Monilinia* spp., helping the growers to speed the decision  
 420 making process about crop protection strategies, to be used in the following growing season.

421

## 422 **Acknowledgments**

423 The research leading to these results has received funding from the European Union's Horizon 2020  
 424 research and innovation program under grant agreement No 634179 "Effective Management of Pests  
 425 and Harmful Alien Species - Integrated Solutions" (EMPHASIS). The authors have no conflicts of  
 426 interest to declare.

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- 592

593 **Tables**

594 **Table 1.** Specificity testing results of the inclusion-exclusion panel of target and non-target species.

595

ISOLATE CODE	SPECIE	ORIGE N	<i>MONILINIA FRUCTICOLA</i> LAMP TEST		<i>MONILINIA LAXA</i> LAMP TEST	
			AVERAGE		AVERAGE	
			Tp (min:s)	Annel °C	Tp (min:s)	Annel °C
1326	<i>Monilinia fructicola</i>	Unknown	07:35±00:17	84.52±0.46	Negative	Negative
C3-29	<i>M.fructicola</i>	Italy	11:05±01:57	84.18±0.16	Negative	Negative
MSR2	<i>M.fructicola</i>	Italy	10:50±01:14	84.07±0.01	Negative	Negative
MSR38	<i>M.fructicola</i>	Italy	09:45±00:15	84.56±0.30	Negative	Negative
MSR47	<i>M.fructicola</i>	Italy	08:00±00:30	84.19±0.08	Negative	Negative
P164C13-12(1)	<i>M.fructicola</i>	Italy	09:25±01:15	84.64±0.74	Negative	Negative
C1C4	<i>M.fructicola</i>	Italy	10:05±00:48	84.76±0.79	Negative	Negative
C14-12 (5)	<i>M.fructicola</i>	Italy	10:15±01:24	84.27±0.1	Negative	Negative
2603	<i>M.fructicola</i>	Italy	10:20±00:23	84.55±0.41	Negative	Negative
MUS26	<i>M.fructicola</i>	Italy	09:15±01:34	84.2±0.12	Negative	Negative
S14CF	<i>M.fructicola</i>	Italy	09:55±01:36	84.14±0.06	Negative	Negative
953	<i>M.fructicola</i>	Unknown	18:35±07:09	84.36±0.21	Negative	Negative
866	<i>M.fructicola</i>	Unknown	14:20± 05:34	84.52 0.36	Negative	Negative
1371	<i>M.fructicola</i>	Australia	10:35±00:23	84.24±0.12	Negative	Negative
853	<i>M.fructicola</i>	Unknown	09:50±02:08	84.42±0.56	Negative	Negative
881	<i>Monilinia laxa</i>	France	Negative	Negative	07:30	82.88±0.45
1402	<i>M. laxa</i>	Italy	Negative	Negative	09:20±00:31	82.44±0.18
1406	<i>M. laxa</i>	Italy	Negative	Negative	10:15±00:40	82.57±0.26
1516	<i>M. laxa</i>	Italy	Negative	Negative	18:45±04:01	82.44±0.24
1757	<i>M. laxa</i>	Italy	Negative	Negative	09:10±01:02	82.54±0.25
1790	<i>M. laxa</i>	Italy	Negative	Negative	07:40±00:31	82.26±0.03

<b>1331</b>	<i>M. laxa</i>	Italy	Negative	Negative	<b>13:30±02:23</b>	<b>83.33±1.66</b>
<b>1368</b>	<i>M. laxa</i>	Australia	Negative	Negative	<b>10:35±00:09</b>	<b>82.62±0.25</b>
<b>1281</b>	<i>M. laxa</i>	Italy	Negative	Negative	<b>10:35±01:57</b>	<b>82.63±0.18</b>
<b>ML1</b>	<i>M. laxa</i>	Italy	Negative	Negative	<b>09:15±01:00</b>	<b>83.29±0.76</b>
<b>887</b>	<i>M. laxa</i>	UK	Negative	Negative	<b>17:50±04:48</b>	<b>82.68±0.74</b>
<b>888</b>	<i>M. laxa</i>	UK	Negative	Negative	<b>17:00±07:26</b>	<b>82.86±0.91</b>
<b>890</b>	<i>M. laxa</i>	UK	Negative	Negative	<b>10:20±01:08</b>	<b>83.08±0.81</b>
<b>1369</b>	<i>M. laxa</i>	Australia	Negative	Negative	<b>08:45±01:09</b>	<b>82.95±0.81</b>
<b>1370</b>	<i>M. laxa</i>	Australia	Negative	Negative	<b>08:30±01:09</b>	<b>82.91±0.61</b>
<b>1767</b>	<i>M. laxa</i>	Unknown	Negative	Negative	<b>09:50±01:17</b>	<b>81.97±0.45</b>
<b>1791</b>	<i>Monilinia fructigena</i>	Unknown	Negative	Negative	Negative	Negative
<b>1756</b>	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
<b>1248</b>	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
<b>1249</b>	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
<b>1515</b>	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
<b>1756</b>	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
<b>48</b>	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
<b>1760</b>	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
<b>1762</b>	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
<b>1763</b>	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
<b>1765</b>	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
<b>1770</b>	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
<b>1773</b>	<i>M. fructigena</i>	Unknown	Negative	Negative	Negative	Negative
<b>1718</b>	<i>Monilinia polystroma</i>	Unknown	Negative	Negative	Negative	Negative
<b>1719</b>	<i>M. polystroma</i>	Unknown	Negative	Negative	Negative	Negative
<b>1538</b>	<i>Verticillium albo-atrum</i>	Unknown	Negative	Negative	Negative	Negative
<b>1540</b>	<i>Colletotrichum truncatum</i>	Unknown	Negative	Negative	Negative	Negative
<b>1542</b>	<i>Helminthosporium solani</i>	Unknown	Negative	Negative	Negative	Negative
<b>1543</b>	<i>Cylindrocarpon</i>	Unknown	Negative	Negative	Negative	Negative
<b>1544</b>	<i>Botryosphaeria berengeriana</i>	Unknown	Negative	Negative	Negative	Negative
<b>1547</b>	<i>Botryosphaeria laricina</i>	Unknown	Negative	Negative	Negative	Negative

1548	<i>B. laricina</i>	Unknown	Negative	Negative	Negative	Negative
2692	<i>Colletotrichum acutatum</i>	Unknown	Negative	Negative	Negative	Negative
92	<i>Zythia fragariae</i>	Unknown	Negative	Negative	Negative	Negative
221	<i>G. cingulata/C. acutatum</i>	Unknown	Negative	Negative	Negative	Negative
256	<i>Colletotrichum acutatum</i>	Unknown	Negative	Negative	Negative	Negative
308	<i>Eutypa lata</i>	Unknown	Negative	Negative	Negative	Negative
332	<i>Macrophoma japonica</i>	Unknown	Negative	Negative	Negative	Negative
452	<i>cylindrocarpon</i>	Unknown	Negative	Negative	Negative	Negative
667	<i>Zythia sp.</i>	Unknown	Negative	Negative	Negative	Negative
668	<i>Mycosphaerella pinodes</i>	Unknown	Negative	Negative	Negative	Negative
677	<i>Lambertella corni-maris</i>	Unknown	Negative	Negative	Negative	Negative
931	<i>Phytophthora fragariae</i>	Unknown	Negative	Negative	Negative	Negative
1138	<i>C.fragariae</i>	Unknown	Negative	Negative	Negative	Negative
1725	<i>Rhizopus spp</i>	Unknown	Negative	Negative	Negative	Negative
2556	<i>Phytophthora fragariae var fragariae</i>	Unknown	Negative	Negative	Negative	Negative
1539	<i>Alternaria spp.</i>	Unknown	Negative	Negative	Negative	Negative
77	<i>Penicillium citrinum</i>	Unknown	Negative	Negative	Negative	Negative
MCAL	<i>Aspergillus flavus</i>	Unknown	Negative	Negative	Negative	Negative
X1	<i>Penicillium expansum</i>	Unknown	Negative	Negative	Negative	Negative
DPO1	<i>Diaporthe spp</i>	Unknown	Negative	Negative	Negative	Negative
BC	<i>Botrytis cinerea</i>	Unknown	Negative	Negative	Negative	Negative
GUN1	<i>Guignardia</i>	Unknown	Negative	Negative	Negative	Negative
PS	<i>Plasmopora</i>	Unknown	Negative	Negative	Negative	Negative
ALTALTER	<i>Alternaria alternata</i>	Unknown	Negative	Negative	Negative	Negative

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**Table 2.** Sensitivity testing of the *M. fructicola* and *M. laxa* LAMP assay reporting the number of replicates amplified out the total replicates tested. In parenthesis the time to positive average of the positive results. The sensitivity testing was carried out using two different isolates.

	<i>M. fructicola</i> isolates		<i>M. laxa</i> isolates	
	MSR38	c14-12	1406	1790
<b>1-10 ng</b>	5/5(07:09)	4/4(09:35)	5/5(11:49)	4/4(08:26)
<b>100-999 pg</b>	5/5(08:00)	4/4(10:35)	5/5(10:41)	4/4(09:37)
<b>10-99 pg</b>	5/5(09:21)	4/4(11:45)	3/5(17:55)	3/4(16:45)
<b>1-9.9pg</b>	3/5(09:56)	1/4(14:30)	1/5(14:15)	0/4
<b>100-999 fg</b>	0/5	0/4	1/5(29:15)	0/4
<b>10-99 fg</b>	0/5	0/4	0/5	0/4
<b>1-9.9fg</b>	0/5	0/4	0/5	0/5

611 **Table 3.** Results of the *M. fructicola* LAMP assay and the qPCR using the primers MI-Mfg-F2 and MI-Mfc-R1 on the DNA obtained from the ‘Fire  
612 Top’ nectarines inoculated with *M. fructicola* and stored at 18°C and 4°C. The LAMP assay was performed on the crude extracted DNA while the  
613 qPCR was carried out using DNA extracted using a commercial kit at different time points (DPI: days post inoculation, Tp: time to positive).  
614

	DPI	Disease Index	LAMP assay			qPCR assay		
			Tp (min:s)	NUMBER OF POSITIVES	ANNEALING TEMPERATURE (°C)	Ct+ DEV.STANDARS (Ct RANGE)	NUMBER OF POSITIVES	CELLS/g
18°C	2	0.00	11:31±01:55 (09:02-14:41)	5/9	84.85±0.07	32.22±1.03 (30.34-33.08)	9/9	3,143.8 (8,477.9-2,000)
	5	0.00	10:03±03:02 (04:33-12:50)	6/9	84.54±0.1	36.39±0.58 (35.69-37.10)	4/9	995.8 (1,439-685.3)
	7	0.67	10:56±00:10 (10:50-11:04)	2/9	85.18±0.01	36.06±2.87 (33.24-39.25)	4/9	759.7 (3,352.9-141.9)
	9	1.00	07:06±04:25 (06:33-07:47)	3/9	84.81±0.14	21.43±8.96 (13.26-36.83)	5/9	5.4+06 (3.98E+08-1,636)
	12	4.00	09:02±01:32 (07:05-11:17)	9/9	85.09±0.1	21.25±6.84 (12.59-30.73)	9/9	6.3+06 (6E+08-42,931)
	14	Not tested						
	16	Not tested						
4°C	2	0.00	18:52±14:48 (09:37-35:57)	3/9	84.43±0.54	33.69±1.09 (32.31-35.30)	7/9	1,559.4 (3,223-668.4)
	5	0.00	12:13±08:21 (07:01-30:59)	8/9	84.56±0.14	Negative		
	7	0.00	12:11±01:25 (11:02-15:00)	6/9	85.17±0.11	38.89±0.78 (38.34-39.44)	2/9	259.7 (346.5-194.2)
	9	0.00	10:28±23 (10:12-10:54)	3/9	84.69±0.17	27.94±5.23 (20.52-33.72)	9/9	2.0+05 (1E+07-9,696)
	12	0.00	19:50±08:54 (13:-36:56)	4/9	84.62±0.83	33.91±1.68 (32.20-36.51)	9/9	6,428.6 (1.6+04 -1,638.9)
	14	1.33	10:23±02:00 (09:08-13:57)	5/9	84.98±0.09	18.61±1.71 (16-20.49)	6/9	7.7E+06 (3E+07-2.8E+06)
	16	0.00	13:49±02:00 (11:11-17:22)	9/9	84.83±0.08	33.82±7.03 (23.49-39.23)	4/9	2,573.7 (5.9E+06-149.6)

**Table 4.** Results of the *M. fructicola* LAMP assay and the qPCR using the primers MI-Mfg-F2 and MI-Mfc-R1 on the DNA obtained from ‘Amiga’ nectarines inoculated with *M. fructicola* and stored at 18°C and 4°C during the time. The LAMP assay was performed on the crude extracted DNA while the qPCR was carried out using DNA extracted using a commercial kit at different time points (DPI: days post inoculation, Tp: time to positive).

	DPI	Disease index	LAMP assay			qPCR assay		
			TP (min:s)	NUMBER OF POSITIVES	ANNEALING TEMPERATURE (°C)	Ct+ DEV.STANDARS (Ct RANGE)	NUMBER OF POSITIVES	CELLS/g
18°C	2	0.00	12:11±01:25 (11:02-15:00)	6/9	85.17±0.11	36.11±1.46 (34.58-39.06)	8/9	28 (62.7-5.9)
	4	1.00	08:18±02:02 (06:35-10:55)	6/9	84.67±0.08	34.23±2.15 (31.99-37)	4/9	66.6 (216.3-15.5)
	7	3.00	08:22±01:38 (07:05-11:46)	9/9	84.95±0.15	22.99±6.47 (16.68-30.98)	6/9	2.2E+04 (6.2 E+05-333.9)
	9	4.00	08:09±00:50 (06:52-09:23)	9/9	84.67±0.13	18.61±1.71 (16-20.49)	6/9	3.9E+05 (1.6E+06-1.46E+06)
	12	5.00	09:51±02:00 (09:57-12:40)	9/9	84.35±0.28	29.88±3.24 (26.75-33.17)	9/9	1,048.5 (5,435.4-185.4)
	14		Not tested					
	16		Not tested					
4°C	2	0.00	14:20±04:19 (11:44-25:31)	9/9	85.0±0.15	36.85±1.85 (33.93-38.33)	8/9	22.8 (106.1-10.5)
	4	1.33	12:14±03:52 (09:50-12:28)	6/9	84.64±0.15	30.33±1.6 (28.26-33.51)	9/9	497.9 (1,482.5-93.6)
	7	0.33	12±01:18 (11:34-15:14)	7/9	85.00±0.12	30.19±2.17 (27.81-33.29)	9/9	626.7 (2,186.9-122.3)
	9	0.00	10:26±00:53 (09:22-11:30)	6/9	84.9±0.08	28.91±2.8 (24.44-32.62)	9/9	1844.3 (1.9E+04-261.5)
	12	1.00	07:13±00:02 06:43-07:426	6/9	84.73±0.14	34.04±4.03 (28.15-39.05)	8/9	123.8 (2,747.3-8.9)
	14	0.67	08±02:03 06:17-11:29	9/9	84.7±0.15	28.15±5.98 (18.6-33.39)	9/9	2754 (4.2E+05-174.4)
	16	1.67	07:25±01:52 (05:52-11:15)	9/9	84.72±0.19	23.7±4.18 (19.43-30.80)	9	2.8E+04 (2.7E+05-681.3)

620

622 **Table 5.** Results of the *M. laxa* LAMP assay and the qPCR using the primers MI-Mfg-F2 and MI-Mfc-R1 on the DNA obtained from the ‘Fire Top’  
623 nectarines inoculated with *M. laxa* and stored at 18°C and 4°C during the time. The LAMP assay was performed on the crude extracted DNA while  
624 the qPCR was carried out using DNA extracted using a commercial kit at different time points (DPI: days post inoculation, Tp: time to positive).  
625

	DPI	Disease index	LAMP assay			qPCR assay		
			TP (min:s)	NUMBER OF POSITIVES	ANNEALING TEMPERATURE (°C)	Ct+ DEV.STANDARS (Ct RANGE)	NUMBER OF POSITIVES	CELLS/g
18°C	2	0.00	Negative			36.36±2.36 (34.69-38.02)	2/9	104.0 (317.4-71.1)
	4	1.33	Negative			Negative		
	7	2.33	12:02±01:39 (10:34-14:23)	4/9	83.55±0.12	36.52±0.84 (35.55-37.03)	3/9	186.9 (358.4-137.7)
	9	4.00	07:52±00:56 (06:45-09:44)	9/9	83.47±0.24	20.66±1.15 (19-22.09)	9/9	2.3E+07 (6E+07-9.9E+06)
	11	5.00	11:13±03:27 (08:29-12:39)	6/9	83.15±0.4	29.37±3.52 (27.17-37.11)	9/9	15E+05 (6,7E+05-418)
	14		Not tested					
	16		Not tested					
4°C	2	0.00	Negative			36.11±1.08 (34.91-37.01)	3/9	126.3 (282.6-139.6)
	4	1.33	Negative			34.45±1.38 (33.47-35.42)	2/9	671.0 (1,289.5-403.9)
	7	0.00	12:46	1/9	83.55	38.32±0.89 (37.69-38.95)	2/9	73.5 (112.1-38.2)
	9	0.00	13:40±08:25 (08:04-28:12)	5/9	83.26±0.15	31.38±1.3 (29.47-32.55)	9/9	1.7E+04 (6.1E+04-9,168.9)
	11	1.00	09:27±00:22 (09:04-09:55)	6/9	83.29±0.16	24.31±6.66 (19.38-34.18)	6/9	2.3E+06 (6E+07-3084.4)
	14	1.33	06:59±04:11 (04:02-11:47)	4/9	83.44±0.07	27.88±2.23 (27.25-30.26)	8/9	5.3 E+04 (2.7E+05-1,2E+04)
	16	1.33	08:07±00:21 (07:46-08:28)	3/9	83.44±0.08	26.6±9.99 (17.52-38.18)	7/9	1.3E+05 (1.8 E+08-54.3)

626



627 **Table 6.** Results of the *M. laxa* LAMP assay and the qPCR using the primers MI-Mfg-F2 and MI-Mfc-R1 on the DNA obtained from ‘Amiga’  
 628 nectarines inoculated with *M. laxa* and stored at 18°C and 4°C during the time. The LAMP assay was performed on the crude extracted DNA while  
 629 the qPCR was carried out using DNA extracted using a commercial kit at different time points (DPI: days post inoculation, Tp: time to positive).  
 630

	DPI	Disease index	LAMP assay			qPCR assay		
			TP (min:s)	NUMBER OF POSITIVES	ANNEALING TEMPERATURE (°C)	Ct+ DEV.STANDARS (Ct RANGE)	NUMBER OF POSITIVES	CELLS/g
18°C	2	0.00	12:29±05:09 (09:00-24:15)	8/9	83.43±0.14	Negative		
	4	1.33	08:44±01:44 (07:16-10:41)	9/9	83.35±0.2	32.4502±3.832 (29.19-39.92)	7/9	81.2 (717.9-0.6)
	7	3.67	08:48±02:44 (08:02-13:20)	9/9	83.25±0.21	28.5379±4.06 (22.81-32.04)	9/9	997.1 (4.6E+04-96)
	9	4.00	07:16±02:28 (04:02-10:00)	9/9	83.35±0.11	29.3679±0.41 (28.97-29.79)	3/9	666.9 (870-502.9)
	12	5.00	08:06±01:13 (06:40-09:50)	9/9	83.27±0.18	25.62±4.09 (20.21-31.58)	9/9	8,151.5 (3E+05-152)
	14	Not tested						
	16	Not tested						
4°C	2	0.00	11:12±01:46 (09:41-13:58)	2/9	83.48±0.1	38.7275±1.07 (37.11-39.93)	5/9	1.3 (3.8-0.6)
	4	0.33	11:30±03:17 (06:59-20:20)	5/9	83.28±0.16	30.9794±2.08 (27.77-33.81)	9/9	188 (1,606.1-28.3)
	7	0.67	10:54±02:17 (08:23-11:55)	5/9	83.28±0.17	25.9199±6.02 (17.79-31.35)	9/9	7,481.7 (1.7E+06-198.5)
	9	1.00	08:55±03:21 (07:38-16:29)	9/9	82.89±1.66	Negative		
	12	1.33	10:04± 06:31 (07:38-16:29)	7/9	83.26±0.13	24.96±1.57 (22.51-26.72)	9/9	1.4 E+04 (7.3E+04-4,382.9)
	14	2.67	07:01±00:17 (05:56-23:09)	9/9	83.47±0.11	24.113±5.9738 (15.28-31.16)	9/9	2.5E+04 (9E+06-225.4)
	16	3.33	0:0:14:01±58 (06:38-07:37)	9/9	83.47±0.11	35.185±2.9199 (30.38-38.35)	6/9	15.3 (379.6-1.8)

**FIGURE LEGENDS**

**Figure 1.** Incidence of *Monilinia* spp. (%) during the monitoring of four winter seasons (2008-2009 to 2011-2012). *Monilinia laxa* (solid line), *Monilinia fructicola* (dashed line), *Monilinia fructigena* (dotted line).

**Figure 2.** Percentage of *Monilinia* spp. isolated from A) nectarine and B) peaches during the winter season 2011-2012.

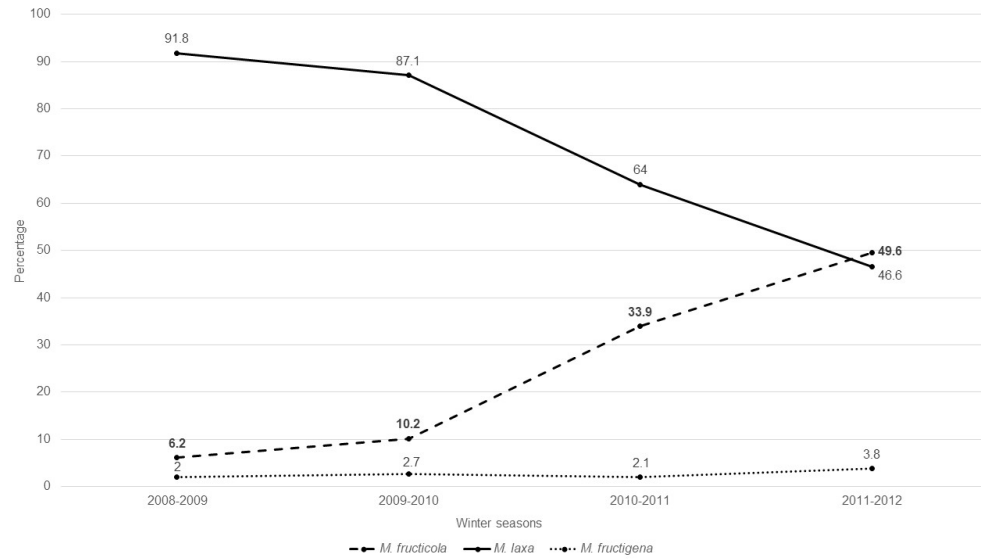


Figure 1. Incidence of *Monilinia* spp. (%) during the monitoring of four winter seasons (2008-2009 to 2011-2012). *Monilinia laxa* (solid line), *Monilinia fruticola* (dashed line), *Monilinia fructigena* (dotted line).

338x190mm (96 x 96 DPI)

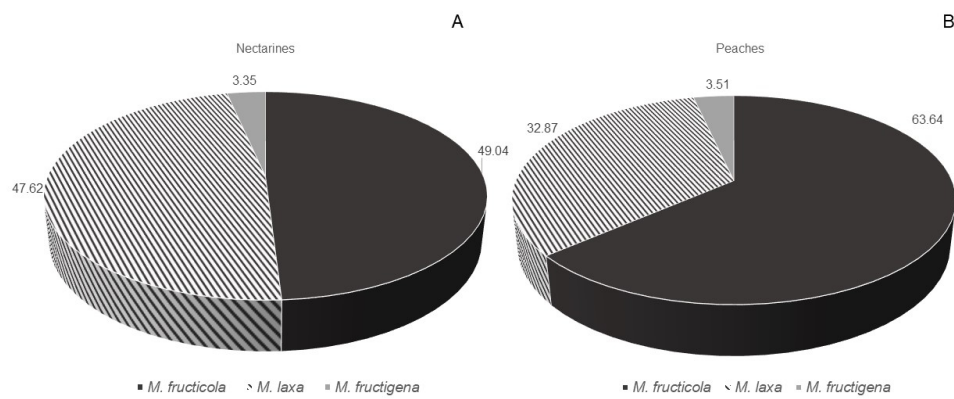


Figure 2. Percentage of *Monilinia* spp. isolated from A) nectarine and B) peaches during the winter season 2011-2012.

338x190mm (96 x 96 DPI)

## Supplementary material

**Table S1.** Orchards monitored during the 2011-2012 winter season with the geographical area, the host, cultivar and percentage of *Monilinia* spp. isolated.

Orchard number	Location	Host	Cultivar	Occurrence of <i>M. fruticicola</i> during			Percentage of different species of <i>Monilinia</i> during 2011-2012		
				2008-2009	2009-2010	2010-2011	<i>M. fruticicola</i> (%)	<i>M. laxa</i> (%)	<i>M. fructigena</i> (%)
1	CASTELLAR	Nectarine	Fire Top				90	0	10
2	COSTIGLIOLE	Nectarine	Big Top	N	N	N	62.5	37.5	0
3	DRONERO	Nectarine	Big Top	N	N	N	0	100	0
4	FOSSANO	Nectarine	Diamond Ray	N	N	N	75	25	0
5	FOSSANO	Nectarine	Diamond Ray	N	N	N	81.25	18.75	0
6	FOSSANO	Nectarine	Big Top	N	N	N	0	100	0
7	LAGNASCO	Nectarine	Big Top	N	N	Y	57.1	42.9	0
8	LAGNASCO	Peach	Vista Rich	N	N	Y	45.55	54.55	0
9	LAGNASCO	Nectarine	Amiga	N	N	N	40	40	20
10	LAGNASCO	Nectarine	Orion	Y	Y	Y	90	10	0
11	LAGNASCO	Nectarine	Big Top	N	N	Y	66.7	33.3	0
12	LAGNASCO	Nectarine	Diamond Ray	N	Y	Y	81.8	18.2	0
13	LAGNASCO	Peach	Red Valley	N	N	Y	54.6	45.5	0
14	LAGNASCO	Nectarine	Big Top	N	Y	Y	88.9	11.1	0
15	LAGNASCO	Nectarine	Diamond Ray	N	N	N	0	76.9	23.1
16	LAGNASCO	Peach	Royal Glory	N	N	N	26.7	66.7	6.6
17	LAGNASCO	Nectarine	Caldesi	N	N	N	0	69.2	30.8
18	LAGNASCO	Nectarine	Fire Sweet Red	Y	Y	Y	90	0	10
19	MANTA	Nectarine	Big Top	N	N	N	0	100	0
20	PIASCO	Nectarine	Big Top	N	N	N	0	100	0
21	REVELLO	Nectarine	Diamond Ray	N	N	Y	100	0	0
22	REVELLO	Nectarine	Venus	N	N	N	25	58.3	16.7
23	REVELLO	Nectarine	Alitop	N	N	Y	100	0	0
24	REVELLO	Nectarine	Venus	N	N	N	46.15	53.85	34

25	SALUZZO	Peach	Elegant Lady	N	N	N	75	12.5	12.5
26	SALUZZO	Nectarine	Nectaross	N	N	N	62.5	37.5	0
27	SALUZZO	Nectarine	Big Top	N	N	Y	100	0	0
28	SALUZZO	Nectarine	Big Top	N	N	N	40	60	0
29	SALUZZO	Nectarine	Venus	N	N	N	62.5	37.5	0
30	SCARNAFIGI	Peach	Rome Star	N	N	N	87.5	0	12.5
31	SCARNAFIGI	Nectarine	Big Top	N	N	N	100	0	0
32	SCARNAFIGI	Peach	Rome Star	N	N	N	100	0	0
33	SAVIGLIANO	Nectarine	V3	N	N	N	0	100	0
34	SAVIGLIANO	Nectarine	Big Top	N	N	N	25	75	0
35	SAVIGLIANO	Peach	Rome Star	N	N	N	16.7	83.3	0
36	SAVIGLIANO	Nectarine	Diamond Ray	N	N	Y	76.5	23.5	0
37	SAVIGLIANO	Peach	Vista Rich	N	N	N	66.7	33.3	0
38	SAVIGLIANO	Nectarine	Amiga	N	N	N	60	40	0
39	VERZUOLO	Nectarine	Stark Red Gold	N	N	N	0	100	0
40	VERZUOLO	Nectarine	Big Top	N	N	N	20	80	0
41	VERZUOLO	Nectarine	Big Top	N	N	N	66.7	33.3	0
42	VERZUOLO	Nectarine	Amiga	N	N	N	0	100	0
43	VERZUOLO	Peach	Rome Star	N	N	Y	100	0	0
44	VERZUOLO	Nectarine	Big Top	N	N	N	20	80	0
45	VERZUOLO	Nectarine	Nectaross	N	N	Y	60	40	0
46	VERZUOLO	Nectarine	Alitop	N	N	N	26.7	60	13.3

**Table S2.** Primers used in this study.

PRIMER	SEQUENCE	TAXA DETECTED	REFERENCE
<b>MO368-5</b>	5'-GCA AGG TGT CAA AAC TTC CA-3'	<i>M. fructigena</i> and <i>M. polystroma</i>	Côté et al. (2004)
<b>MO368-8R</b>	5'-AGA TCA AAC ATC GTC CAT CT-3'		
<b>MO368-5</b>	5'-GCA AGG TGT CAA AAC TTC CA-3'	<i>M. fructicola</i>	Côté et al. (2004)
<b>MO368-10R</b>	5'-AAG ATT GTC ACC ATG GTT GA-3'		
<b>MO368-5</b>	5'-GCA AGG TGT CAA AAC TTC CA-3'	<i>M. laxa</i>	Côté et al. (2004)
<b>LAXA-R2</b>	5'-TGC ACA TCA TAT CCC TCG AC-3'		
<b>ITS1</b>	5'-TCC GTA GGT GAA CCT GCG G-3'	<i>M. fructigena</i>	White et al. (1990)
<b>MFG-R2</b>	5'-GGT CAA CCA TAG AAA ATT GGT-3'		Hughes et al., (2000)
<b>MCF-F1</b>	5'-TAT GCT CGC CAG AGG ATA ATT A-3'	<i>M. fructicola</i>	Hughes et al. (2000)
<b>MFC-R1</b>	5'-GAT TTT AGA GCC TGC CAT TA-3'		
<b>MI-MFG-F2</b>	5'-GCT CGC CAG AGA ATA ATC-3'	<i>M. laxa</i>	Hughes et al. (2000)
<b>MI-MFC-R1</b>	5'-GAT TIT AGA GCC TGC CAT TG-3'		
<b>IGENAS</b>	5'-TGCTCTGCCCCGTACCCAG-3'	<i>M. fructigena</i>	Gell et al. (2007)
<b>IGENAAS</b>	5'-GGATTTATTGTGATGTAGTTTCG-3'		
<b>ICOLAS</b>	5'-GAGACGCACACAGAGTCAG-3'	<i>M. fructicola</i>	Gell et al. (2007)
<b>ICOLAAS</b>	5'-GAGACGCACATAGCATTGG-3'		
<b>ILAXAS</b>	5'-TGAGCACGAGTGAATGTATAG-3'	<i>M. laxa</i>	Gell et al. (2007)
<b>ILAXAAS</b>	5'-TGAGCACGAGGGCATATC-3'		

**Figure S1.** A. Percentage of *M. fruticola*, *M. laxa* and *M. fructigena* in function of the A. *Prunus persica* cultivars during the 2011-2012 winter season. B. A. Geographical origin during the 2011-2012 winter season.

